SUBUNIT INTERACTIONS IN ASPARTATE TRANSCARBAMYLASE FROM *ESCHERICHIA COLI* STUDIED USING MATRIX-BOUND DERIVATIVES

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1. Introduction

The relationship between the structure and function of asparate transcarbamylase (ATCase) from *Escherichia coli* is of particular interest to enzymologists because of the well-known homotropic and heterotropic interactions which exist in this enzyme. The sigmoidal saturation curve for the substrate aspartate and the effects of CTP and ATP respectively in enchancing and reducing the sigmoidicity have been well characterized (for reviews see refs. [1,2]. There is also considerable information on the structure of ATCase particularly with regard to the number of subunits and their arrangement in the native enzyme. The important role of subunit interactions on the allosteric properties of ATCase is clearly indicated by the observation that dissociation of the native enzyme into the two types of subunits is accompanied by the loss of both the homotropic and the heterotropic effects [3].

In previous work from this laboratory, we have studied the effects of subunit interactions in oligomeric enzymes by comparing the properties of subunit derivatives with those of the intact enzyme. The experimental methods of approach used were: (a) by binding subunits covalently to a matrix such as Sepharose to prevent their association [4–7]; (b) by studying the subunits as intermediates formed during renaturation at high dilution [8], and (c) by chemical modification at the intersubunit surface [9]. We are now applying these methods to analyze the subunit interactions responsible for the allosteric mechanism of ATCase. Using approach (b) described above, we have previously studied the properties of a derivative of ATCase with an incomplete quaternary structure formed by adding excess regulatory subunits to a very dilute solution of the catalytic subunit [10]. In this paper, we report the results of parallel studies using matrix-bound derivatives.

2. Experimental

The reagents used and the methods for the preparation and measurement of matrix derivatives were essentially the same as described previously [5,6]. Aspartate transcarbamylase was purified according to Gerhart and Holoubek [11] and the Zn regulatory subunit was prepared by the procedure of Nelback et al. [12].

ATCase activity was measured with an automatic titrator using a modification of the method described by Wu and Hammes [13]. The assay mixture (2 ml) consisted of 0.1 M potassium acetate, 20 mM 2-mercaptoethanol, 100 μg bovine serum albumin, 5 mM carbamyl phosphate and 6 mM potassium aspartate (unless otherwise specified). The pH was adjusted to 8.5 and the temperature maintained at 25°C. The reaction was started by addition of the enzyme and was monitored by titration with 40 mM sodium hydroxide containing 40 mM aspartate (which had previously adjusted to pH 8.5). The inclusion of aspartate in the titrant was necessary to compensate for the depletion of aspartate during the assay since the concentration of this substrate was not at a saturating level. Adequate stirring was maintained to ensure good mixing and continuous suspension of matrix-bound derivatives. The rate of base-uptake was corrected for the small background rate due to spontaneous hydrolysis of carbamyl phosphate [14]. A unit of activity is defined

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as the amount of enzyme converting one μmole of aspartate into carbamyl aspartate per minute at 25°C.

Sepharose 4B (2 ml packed volume) was activated with cyanogen bromide (2 mg, freshly dissolved in 80 μl water) in a thick slurry (3 ml total volume) according to Axén et al. [15]. After washing quickly with 25 ml cold potassium pyrophosphate (0.1 M, pH 8.5), the activated Sepharose was drained from excess liquid and then transferred to 0.63 ml of the same pyrophosphate buffer containing 10 mg ATCase. After allowing coupling of the enzyme to proceed for 2 hr at room temperature, the material was filtered and transferred to 5 ml ethanolamine–HCl (0.1 M, pH 8.5) containing 1 mM EDTA and kept at 4°C for 72 hr. The product was washed seven times with a total of 200 ml potassium phosphate buffer (40 mM, pH 7.0) containing 1 M NaCl, 2 mM 2-mercaptoethanol and 0.2 mM EDTA. The bound-ATCase so prepared was stored in the same buffer as used in the final washing step except that NaCl was omitted.

A portion of the bound-ATCase (2 ml of 1 in 10 suspension) was washed and resuspended in 2 ml potassium phosphate buffer (40 mM, pH 7.0) and dissociation into subunits was initiated by adding 12.6 mg *p*-hydroxymercuribenzoate (PHMB) in 0.3 ml Tris–HCl buffer (0.1 M, pH 8.5). The mixture was incubated at 25°C with stirring for 2 hr. The product was washed five times at 4°C with a total of 25 ml imidazole–HCl buffer (10 mM, pH 7.0) containing PHMB (50 μM) and NaCl (1 M). Then 2-mercaptoethanol and EDTA were added to final concentrations of 20 mM and 50 mM respectively and left for 15 min at 4°C with stirring. It was then washed three times with a total of 15 ml potassium phosphate buffer (40 mM, pH 7.0) containing 2-mercaptoethanol (2 mM) and EDTA (5 mM) then five times with a total of 25 ml of the same buffer but without EDTA. This derivative is referred to in this paper as bound subunit-ATCase.

4. Results and discussion

Native ATCase is known to consist of six polypeptides containing catalytic sites and six polypeptides containing regulator binding [16] sites. The catalytic polypeptides (*c*) are arranged as trimeric subunits (c_3) and the regulatory polypeptides (*r*) are arranged as dimeric subunits (r_2). In fig. 1, ATCase is represented as two c_3 particles linked non-covalently via three r_2 bridges [17,18] and coupled covalently to Sepharose via either a catalytic or a regulatory subunit. Since only very low amounts of cyanogen bromide (1 mg/ml Sepharose) were used to activate Sepharose, most of the molecules are probably coupled via only one subunit [4]. Treatment of this derivative with PHMB is known to dissociate the enzyme into c_3 and r_2 particles and extensive washing of the treated derivative should result in bound subunit-ATCase which presumably contains both c_3 and r_2 which are unable to interact with each other. Addition of soluble r_2 subunits should then lead to the formation of bound c_3r_6 complex. This derivative should show no tendency to convert to the native enzyme (c_6r_6) since this process would require the participation of two c_3 particles bound to different parts of the matrix.

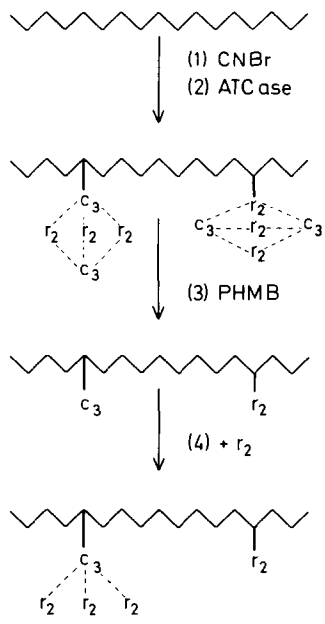

Fig. 1. Scheme showing the preparation of bound-ATCase and bound subunit-ATCase. ^^^ denotes the matrix of Sepharose 4B; c_3 and r_2 represent respectively the catalytic and the regulatory subunits of ATCase; covalent linkages are indicated by solid lines and non-covalent linkages by broken lines.

Bound-ATCase shows a sigmoidal substrate saturation curve similar to that of the native enzyme in solution and is also inhibited by CTP and activated by ATP. The activity of bound subunit-ATCase on the other hand resembles that of the isolated catalytic subunit and is insensitive to CTP or ATP. Details of these results will be published elsewhere (Chan, W. W.-C., in preparation). In converting bound-ATCase to the corresponding subunit derivative, the activity (measured at pH 8.5 and 6 mM aspartate) decreased from 44 units/ml Sepharose to 7 units/ml. If we assume that bound-ATCase and the bound subunits have the same specific activities as in free solution (33 U/mg for ATCase and 210 U/mg for c_3 at pH 8.5 and 6 mM aspartate) and that dissociation occurred as shown in fig. 1, then we can calculate that out of approximately 1.3 mg ATCase originally bound to each ml of Sepharose only 0.1 mg was bound via the catalytic subunit. The r_2 in the bound subunit-ATCase derivative (estimated to be 0.14 mg/ml) should however have no effect on subsequent experiments since only activity was measured.

Studies with highly diluted c_3 solutions have shown that upon addition of excess r_2 to form c_3r_6, a 2-fold increase occurs in the activity at pH 8.5 and 6 mM aspartate [10].

Recently we have characterized the c_3r_6 complex as having a sedimentation coefficient (7.7 S) intermediate between those of the native enzyme (11.7 S) and of c_3 (5.8 S) and have shown that the formation of c_3r_6 is reversible.*

Upon the addition of excess r_2 to bound subunit-ATCase, there is also an approximately two-fold increase in the activity at 6 mM aspartate. However, when the amount of r_2 added is not in great excess, the behaviour of bound subunit-ATCase is markedly different from that of c_3 in solution. Thus at non-saturating levels of r_2, the activity of soluble c_3 increases initially due to formation of the c_3r_6 complex and then decreases as the complex is gradually converted to the native enzyme [10]. In the case of bound subunit-ATCase on the other hand, the c_3r_6 complex formed shows no tendency to be converted to the native enzyme and the assay remains linear at all levels of r_2. Thus, as expected, the matrix appears to be able to prevent the reassociation into the native c_6r_6 structure. This property of bound subunit-ATCase makes it suitable for the study of the reversible process: bound-c_3 + 3 r_2 $\rightleftharpoons$ bound-c_3r_6, since no complication arises from the conversion to the native enzyme. This process was followed by measuring the increase in activity ($v - v_0$) due to the formation of c_3r_6 at different levels of r_2. The resulting r_2-saturation curve is a simple hyperbola and the double reciprocal plot $1/(v - v_0)$ versus $1/[r_2]$ is given in fig. 2. The horizontal intercept gives the negative

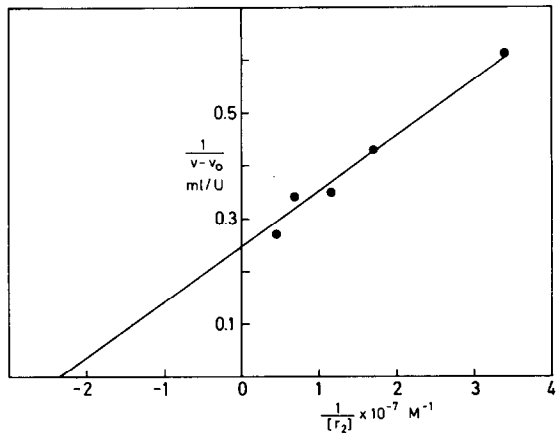

Fig. 2. Double reciprocal plot of the increase in activity ($v - v_0$) versus the concentration of r_2. The sample of bound subunit-ATCase used for each measurement consisted of 50 µl of a 1 in 10 suspension. Conditions of the assay are described in the Experimental section.

reciprocal of the r_2 concentration for half-saturation. This r_2 concentration is estimated to be about 1.5 µg r_2/ml or 0.044 µM. From the simple hyperbolic nature of the saturation curve, it is probably safe to assume that each r_2 binds independently to one of three sites on c_3 and that the increase in activity observed is proportional to the number of r_2 subunits bound. It can then be shown that the association constant K_{assoc} is equal to the reciprocal of r_2 concentration for half-saturation. From this value of K_{assoc} (2.3×10^7 M^{-1}) the free-energy change for c:r interaction works out to −10 kcal/mole.

Recently it has also been possible to measure the K_{assoc} of the above process in free solution by working

* Mort, J. S. and Chan, W. W.-C., manuscript submitted for publication.

at extremely low concentrations of c_3 (nanogram level) to prevent conversion to the native enzyme and the K_{assoc} so determined was very similar to that above. It was also possible to show that CTP and ATP had no significant effect on the *c:r* interaction in the c_3r_6 complex.** These results together with the similarity of the c_3r_6 complex to the relaxed (*R*) state of the enzyme and its insensitivity to CTP and ATP [10], see also footnote*, suggests that the heterotropic effects of CTP and ATP operate primarily by affecting the *r:r* domain. A model of the allosteric mechanism based on these results will be published elsewhere.**

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